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Resistance to Pseudorabies Virus Infection in Transgenic Mice Expressing the Chimeric Transgene That Represses the Immediate-Early Gene Transcription

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A chimeric gene encoding a fusion protein consisting of the DNA-binding domain of the immediate-early (IE) protein of pseudorabies virus (PRV) and a tail-truncated VP16 of herpes simplex virus 1, lacking the transcription activation domain, has been shown to repress transcription of the PRV IE gene, resulting in the inhibition of PRV growth *in vitro*. To assess the antiviral potential of the fusion protein *in vivo*, transgenic mice containing the chimeric gene under the control of the virus- and interferon-inducible Mx 1 promoter were generated. A transgenic mouse line showed marked resistance to PRV infection when the mice were challenged intranasally with PRV. Inhibition of PRV replication was also observed in monolayers of embryonic cells prepared from the transgenic mice. In the cells infected with PRV, transcription of the PRV IE gene was repressed. The present results indicate that the chimeric gene is able to exert a significant antiviral effect against PRV infection *in vivo*.

INTRODUCTION

Pseudorabies virus (PRV) of genus *Varicellovirus* of the subfamily *Alphaherpesvirinae* (Roizman, 1990) causes severe disease in piglets and leads to latent infection in all surviving pigs. PRV infection inflicts serious losses on the swine industry worldwide. It is also known that PRV causes acute and often fatal infection in domestic and wild animals.

PRV expresses a single immediate-early (IE) protein species from two copies of the IE gene that are present in each inverted repeat region of the viral genome (Ihara *et al.,* 1983). The coding region of the IE gene is 4380 nucleotides long and codes for 1460 amino acid residues (Cheung, 1989). The product of PRV IE gene, IE180, has been shown to be a homology of one of the IE gene products, ICP4, of herpes simplex virus 1 (HSV-1) (Cheung, 1989). IE180 functions for continuous transcription of late genes and in shutting off the synthesis of their own RNA (Kit, 1994), indicating that the IE gene is absolutely necessary for productive lytic infection.

"Intracellular immunization" is proposed as an approach to antiviral therapy in humans and to germ-line transformation in animals to confer resistance to virus infection (Baltimore, 1988). Intracellular immunization against HSV-1 infection *in vivo* has been shown by using a mutated form of ICP4 (Smith and DeLuca, 1992). For the purpose of intracellular immunization against pseudorabies, we have set our sights on inhibition of the PRV IE gene expression. We have shown that the chimeric gene product (Ono *et al.,* 1995) and the dominant-negative mutants of IE180 (Taharaguchi *et al.,* 1994; Ono *et al.,* 1998a) and early protein 0 (EP0) (Watanabe *et al.,* 1996; Tasaki *et al.,* unpublished data) can repress transcription of the PRV IE gene. Among them, the chimeric gene was most effective for the purpose. The chimeric gene encodes a fusion protein consisting of the DNA-binding domain of IE180 of PRV and a tail-truncated VP16 of HSV-1, lacking the transcription activation domain. Marked inhibition of PRV replication in the cell lines transformed with the chimeric gene has been shown (Ono *et al.,* 1995). The mechanism via which the IE gene transcription was inhibited with the fusion protein was thought to be binding of the fusion proteins to the consensus pentanucleotides (5′-ATCGT-3′) and/or the octamer sequences on the IE promoter by each binding domain originating from IE180 and VP16, and the fusion protein sterically interfered with the formation of the transcription machinery or movement of RNA polymerase II.

In the present study, a transgenic mouse line expressing the chimeric gene that represses the PRV IE gene transcription was established, and resistance to PRV

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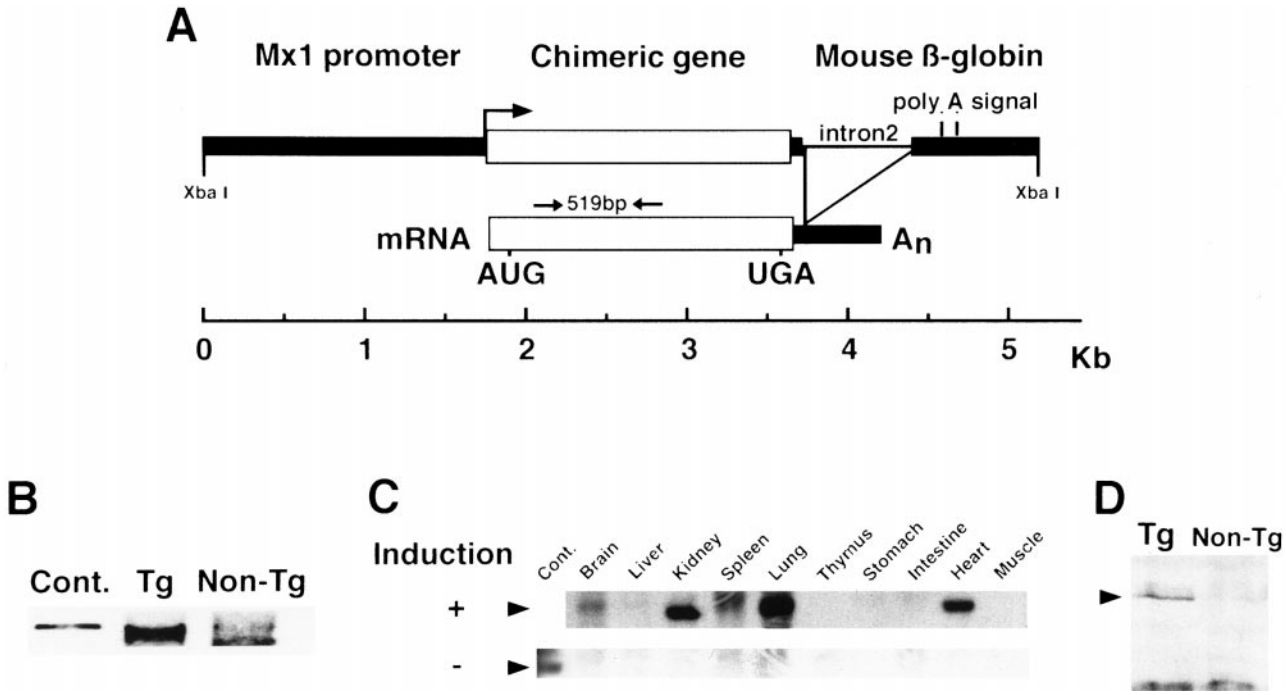

FIG. 1. (A) Schematic representation of the transgene fragment. The chimeric gene is under the control of the Mx 1 promoter, which can be induced by interferon, double-stranded RNA, or virus infection. Location of the primers used for RT-PCR of the chimeric gene mRNA and expected size of amplified fragment are shown. (B) Southern blot analysis of tail DNA from transgenic and nontransgenic mice. The first lane was loaded with 100 pg of *Bam*HI-digested pssRGD. The last two lanes were loaded with 10 μg of *Bam*HI-digested mouse DNA. (C) RT-PCR analysis of the chimeric gene mRNA in various tissues of a noninduced transgenic mouse and a transgenic mouse induced with double-stranded RNA for 18 h. PCR products were analyzed as in B by Southern blot analysis. (D) Western blot analysis of total lung extracts from transgenic and nontransgenic mice treated as in C.

infection in the transgenic mice was examined to determine the antiviral potential of the chimeric gene *in vivo*.

RESULTS

Characterization of transgenic mice

To generate transgenic mice, DNA fragments containing the chimeric gene were microinjected into C57BL/6 fertilized eggs. In these transgenic mice, the chimeric gene was expressed under the control of the mouse Mx 1 promoter, which can be induced by double-stranded RNA, interferon, or virus infection (Fig. 1A). Of the resulting 33 births, four animals had the transgene as determined by Southern blot analysis of tail DNA. In these founder mice, one founder had more than five copies of the transgene per haploid DNA (Fig. 1B). To examine tissue specificity of the transgene expression, mRNA of the chimeric gene in a variety of organs from the transgenic mice was detected by the RT-PCR method. The transgene expression was observed in brain, kidney, lung, and heart (Fig. 1C). In the total lung extract prepared from the transgenic mice after intraperitoneal injection of poly(I)–poly(C), anti-HSV-1 VP16 monoclonal antibody-specific band was detected by Western blot analysis (Fig. 1D). The other three founders failed to transmit the transgene to their progeny, presumably due to mosaicism (Wilkie *et al.,* 1986).

In the course of breeding the transgenic line, we observed that the F1 transgenic mice consistently weighted less than their nontransgenic littermates. The weight difference was particularly pronounced after weaning (3 weeks), although the transgenic mice could be sorted between birth and weaning on the basis of visual determination of size (Fig. 2). This difference was statistically significant and independent of sex. In addition to the phenotypical dwarf, their reproductive performance was very poor, and the ratio of females at birth was high, suggesting that the presence of the chimeric

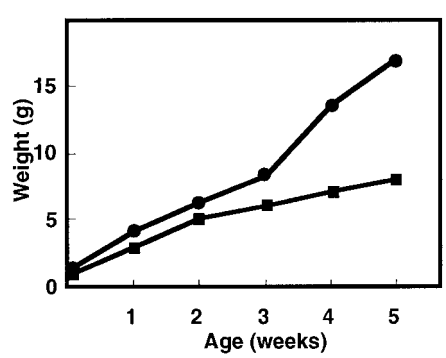

FIG. 2. Retarded growth of transgenic mice. Growth curve of transgenic (■) and nontransgenic (●) mice. The values are the averages of seven each of transgenic and nontransgenic mice at the indicated time.

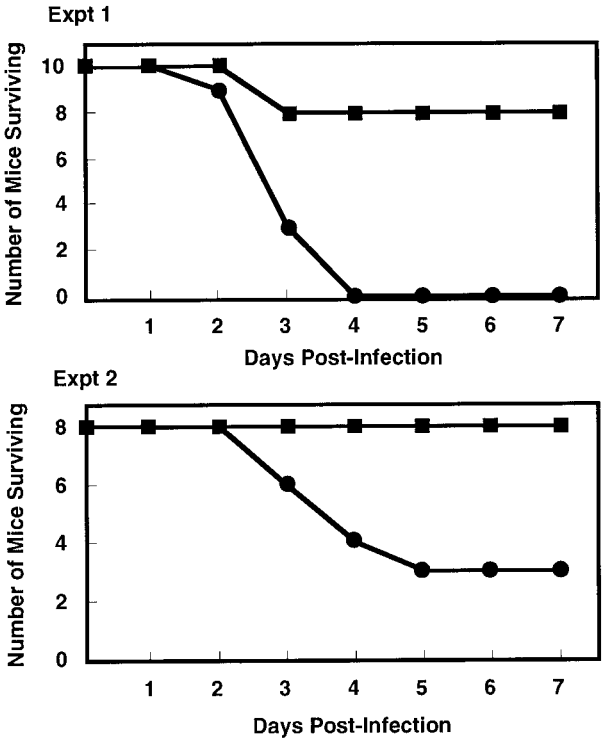

FIG. 3. Resistance to PRV infection in transgenic mice. Transgenic (■) and nontransgenic (●) mice were challenged intranasally with PRV. Protection tests were performed in duplicate (Expts. 1 and 2). The mice were observed for their survival times, which are recorded daily for 7 successive days after the challenge.

gene elicits negative effects during embryogenesis and/or after birth.

Resistance of the transgenic mice to PRV infection

To determine whether the transgenic mice expressing the chimeric gene were protected from PRV infection, 2 LD_{50} of PRV was intranasally inoculated into transgenic and nontransgenic mice. The survival data (Fig. 3) indicated that the transgenic mice were resistant to PRV. All of control mice in experiment 1 and 5 of 8 control mice in experiment 2 died within 5 days after the virus infection. In contrast, 8 of 10 transgenic mice (experiment 1) and all of transgenic animals (experiment 2) survived the viral inoculation and remained healthy for several months after this trial. Five of the surviving transgenic mice and all of the surviving control mice were sacrificed 14 days after the challenge, and the sera were examined for antibody titer against PRV. Specific antibodies to PRV in the samples were detected in all of the surviving transgenic mice and the titers were 1:200 to 1:1600, indicating that the virus replication occurred in the transgenic mice and that the mice were protected from the viral infection. In contrast, specific antibodies were not detected in the sera of surviving nontransgenic mice, indicating that the mice were not infected with the virus.

Suppression of the growth of PRV in cultured embryonic fibroblasts from the transgenic mice

To assess whether the *in vivo* resistance to PRV infection in the transgenic mice was paralleled by resistance of their isolated cells, we tested embryonic fibroblasts for resistance to PRV infection in culture. Transgenic and nontransgenic fibroblasts treated with interferon were infected with 0.01 PFU/cell of PRV. To monitor the virus growth in these fibroblasts, the virus titers in the media were determined (Fig. 4A). In transgenic fibroblasts, the virus yield was extensively suppressed (100-fold less in virus titer than for nontransgenic fibroblasts 4 days postinfection). However, there was no difference between transgenic and nontransgenic fibroblasts without interferon treatment (Fig. 4A). To test whether transcription of the IE gene is inhibited during PRV infection, accumulation of IE mRNA in the PRV-infected and cycloheximide-treated cells was examined by Northern blot analysis. As shown in Fig. 4B, transgenic fibroblasts produced less IE mRNA than did control fibroblasts. The decrease in the IE mRNA level was not due to differences in the loading of the samples as demonstrated by β-actin hybridization of the same filter. In fibroblasts derived from transgenic embryos, expression of the fusion protein was induced by treatment with interferon (Fig. 4C). These results indicate that the impediment of lytic infection of PRV in transgenic fibroblasts is mediated at least in part by inhibition of the PRV IE gene transcription by the fusion protein.

DISCUSSION

Pseudorabies inflicts a major economic loss in pig industries worldwide. Vaccination of pigs against PRV infection with modified-live virus or inactivated virus induces neutralizing antibodies and sensitized lymphocytes in blood and lymphoid organs. Currently available vaccines suppress manifestation of the disease but do not confer complete protection against the virus infection. New strategies such as "intracellular immunization" should therefore be tried to eradicate the disease. In the present study, transgenic mice expressing the chimeric gene that represses the PRV IE gene transcription showed significant resistance to PRV infection. This resistance was much more striking than that observed in HSV-1-resistant transgenic mouse lines expressing a mutant allele (X25) of ICP4 (Smith and DeLuca, 1992) and in PRV-resistant transgenic mouse lines expressing the human interferon-β1 (Chen *et al.,* 1988). The results of the present study therefore suggest the potential value of the chimeric gene in pigs for intracellular immunization against PRV infection.

Consistent with an earlier study (Ono *et a*l., 1995), transcription of the PRV IE gene was repressed in the transgenic embryonic fibroblasts infected with PRV, resulting in the impediment of lytic infection of PRV. These

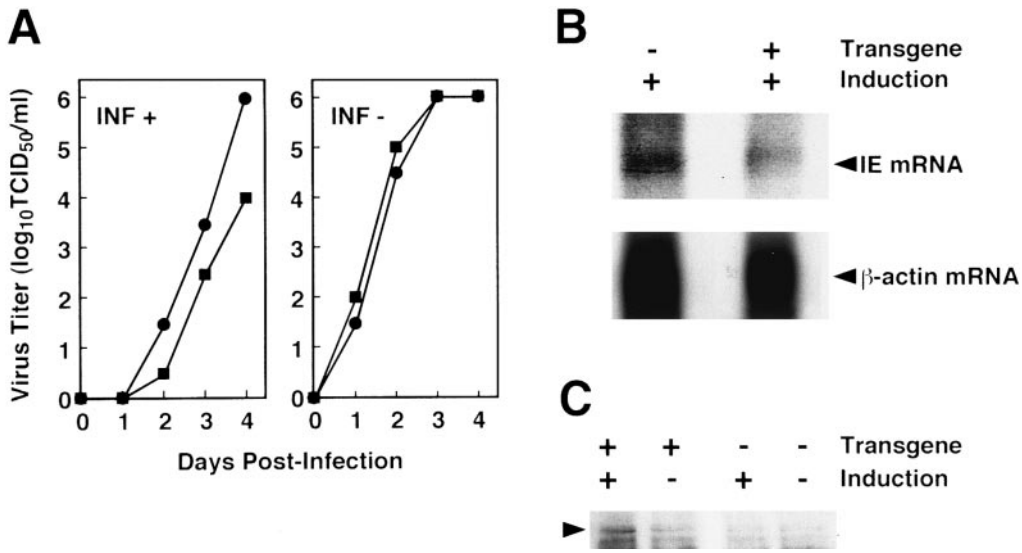

FIG. 4. Resistance to PRV infection in embryonic fibroblasts prepared from transgenic mice. (A) PRV growth curves in embryonic fibroblasts prepared from transgenic (■) and nontransgenic (●) mice. The virus yields in the interferon-treated (IFN+) and untreated (IFN−) fibrloblasts were determined on Vero cell monolayers. (B) Northern blot analysis of IE mRNA synthesized in embryonic fibroblasts prepared from transgenic and nontransgenic mice. Positions of detected IE and β-actin mRNA are indicated on the right. (C) Western blot analysis of nuclear extracts from transgenic and nontransgenic fibroblasts treated with interferon and noninduced transgenic and nontransgenic fibroblasts. Position of the detected fusion protein is indicated on the left.

results indicate that the resistance of transgenic mice to PRV infection is mediated at least in part by inhibition of the PRV IE gene transcription by the fusion protein. In the present study, transgenic mice were treated with poly (I)–poly(C) before the viral challenge. The treatment may affect the viral replication in the transgenic mice. In Vero cells treated with interferon-α, transcription from the PRV IE promoter was repressed and PRV replication was suppressed (Tonomura *et al.,* 1996). Furthermore, *in vivo* studies indicated that interferons had important roles for resistance to PRV infection in mice (Chen *et al.,* 1988; Onodera *et al.,* 1994). Although the susceptibility of nontransgenic mice to PRV infection did not change in our experiments, we cannot exclude a possibility that the immune system induced by the poly(I)–poly(C) treatment is involved in the observed antiviral activity in the transgenic mice. Because the specific antibodies to PRV were detected in the surviving transgenic mice, the protection from PRV infection in transgenic mice was not considered to be complete in the sites of primary infection. These findings suggest a possible explanation for the resistance to PRV infection in transgenic mice. The fusion proteins repress the PRV IE gene transcription in the sites of primary infection, resulting in suppression of the viral replication. Because the viral replication is suppressed, spread of the virus in transgenic mice is delayed. In the meantime, the host immune system [including interferon induced by the poly(I)–poly(C) treatment and/or PRV infection] is evolved to provide protection from PRV infection. Consequently, transgenic mice survive the PRV challenge.

It appears that the chimeric gene may exert adverse side effects in addition to conferring the desirable characteristic of PRV resistance. First, the transgenic mice consistently weighed less than the nontransgenic mice. Similar findings were observed in the transgenic mice expressing the dominant-negative repressor of ICP4 of HSV-1 (Smith and DeLuca, 1992). Second, their reproductive performance was very poor and the ratio of females at birth was high. Finally, the expression level of the chimeric gene was very low, which is consisted with the earlier *in vitro* study (Ono *et al.,* 1995). Lesser amounts of dominant-negative repressor in the transfected cells were reported in other viral regulatory proteins (Spatz *et al.,* 1996; Ono *et al.,* 1998a). It is considered that dominant-negative repressors are simply toxic to the cells and that the expressions are tightly controlled through a negative autoregulatory mechanism. To eliminate the side effects, a tightly controlled expression system (including tissue and/or cell specificity, expression time, and level) would be necessary. If achievable, the generation of pseudorabies-resistant animals will be possible.

The surviving transgenic mice had specific antibodies to PRV. This finding may indicate that the transgenic mice are latently infected with PRV, although latent infection in the transgenic mice was not examined. The reactivation mechanism of latently infected PRV has not been elucidated. It has been shown in HSV-1 that ICP0 is required for the viral reactivation in trigeminal ganglia (Cai *et al.,* 1993; Leib *et al.,*1989; Zhu *et al.,* 1990). EP0 of PRV has been reported by Cheung (1991) to be a homolog of ICP0 of HSV-1. EP0 is a viral transactivator of PRV (Watanabe *et al.,* 1995) and may be important for reactivation from the latent state (Cheung, 1996). EP0 enhances the PRV IE

gene transcription (Watanabe *et al.,* 1995) and the infectivity of PRV genomic DNA (Ono *et al.,* 1998b). EP0 is, however, nonessential for PRV replication (Cheung *et al.,* 1994), whereas IE180 is essential (Ihara *et al.,* 1983). It is therefore conceivable that IE180 is essential for PRV replication from the latent state, although EP0 may have an important role or roles for the first step of reactivation to activate the IE gene. Because the fusion protein represses PRV IE gene transcription, reactivation from the latency would be repressed when the chimeric gene is expressed in the trigeminal ganglia. The repression would occur even when EP0 was expressed in the first step of reactivation because transcription of the IE gene was repressed in cotransfection experiments with the EP0- and the chimeric gene-expression plasmids (Ono *et al.,* unpublished data). The generation of transgenic mice repressing PVR IE gene transcription in the trigeminal ganglia would make it possible to prevent reactivation from latent infection and hence control the spread of PRV throughout a population.

MATERIALS AND METHODS

Virus and cells

PRV strain YS-81 and a cellular clone of the porcine kidney cell line (CPK) were kindly provided by M. Shimizu (National Institute of Animal Health, Tsukuba, Japan). Vero cells were a generous gift from M. Peeples (Rush University, Chicago, IL). CPK cells, grown in Eagle's minimum essential medium supplemented with 10% calf serum, 0.03% L-glutamine, 0.03% tryptose phosphate broth, nonessential amino acids (Flow Laboratories, Costa Mesa, CA), vitamins (Flow Laboratories), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, were used for propagation of the virus. Vero cells, grown in RPMI 1640 medium supplemented with 5% FBS, 0.03% L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, were used to titer the virus as described previously (Ono *et al.,* 1995).

Embryonic fibroblasts were isolated by dicing 14-day-old mouse embryos, stirring the pieces for 30 min at room temperature in PBS, supplementation with 0.25% trypsin, and then plating out the resulting single cell suspension. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS and the above antibiotics. The cells were passed no more than twice before use.

Transgenic mice

The *Bam*HI linker was inserted into the *Hin*dIII site end-filled with a large fragment of DNA polymerase I to obtain a *Bam*HI fragment encoding the fusion protein from pcRGD (Ono *et al.,* 1995). Plasmid pSS-2-2 (Mitchell *et al.,* 1993) containing the Mx 1 promoter (Hug *et al.,* 1988) and the mouse β-globin sequence (Konkel *et al.,* 1978) cloned into pSP65 was linealized at the single *Bam*HI site at the junction between the promoter and the β-globin sequence. The linealized plasmid and the *Bam*HI fragment containing the chimeric gene were ligated. The resulting plasmid was designated pssRGD. The 5.2-kb *Xba*I fragment (Fig. 1A) from the construct was isolated and purified, and approximately 500 copies were microinjected into the pronuclei of fertilized C57BL/6 mouse embryos. They were transplanted into the oviducts of pseudopregnant females as described by Yamamura *et al.* (1984).

Transgenic founders were identified by Southern blot analysis (Southern, 1975) using genomic DNA isolated from mouse tail (Hogan *et al.,* 1986). The DNA samples (10 μg) were digested with *Bam*HI, fractionated on 0.8% agarose gel, and transferred to a Hybond-N^+ membrane (Amersham Life Science, Buckinghamshire, England). Digoxigenin (DIG)-labeled oligoprobes for detection of the transgene were derived from a 1.8-kb *Hin*dIII–*Bam*HI fragment of pcRGD using a DIG DNA labeling kit (Boehringer Mannheim Biochemica, Mannheim, Germany). Hybridization and detection of the transgene were performed using a DIG nucleic acid detection kit and CSPD (Boehringer Mannheim Biochemica) as directed by the manufacturer except that hybridized membranes were washed at 68°C instead of 42°C.

Analysis of transgene expression

Transgenic mice were injected intraperitoneally with 50 μg of poly(I)–poly(C) (Wako Pure Chemical Industries, Osaka, Japan) or left untreated. Eighteen hours after the induction, total RNA was isolated from various tissues of the transgenic mice using TriZOL reagent (GIBCO BRL). To detect the chimeric gene-specific mRNA, the total RNA was amplified by RT-PCR to obtain a fragment of HSV-1 VP16 coding region. The cDNA was synthesized from 10 μg of the total RNA by Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) using oligo$(dT)_{18}$ as a primer at 42°C for 1 h. The primers used to identify the transgene expression were 5′-CTATCGAACCGTGTTGGCCAACTT-3′ and 5′-ATCAACACCATAAAGTACCCAGAG-3′, which resulted in the generation of a 519-bp HSV-1 VP16 cDNA fragment (Fig. 1A). PCR was performed by 30 reaction cycles. A single cycle consisted of three reactions: denaturation at 94°C for 1 min, annealing at 58°C for 3 min, and extension at 72°C for 3 min. The PCR products were fractionated on 1% agarose gel and analyzed by Southern blot analysis as described above.

Western blotting was performed according to the method of Towbin *et al.* (1979). Transgenic and nontransgenic mice were injected intraperitoneally with 50 μg of poly(I)–poly(C). Eighteen hours after the induction, total lung extracts were prepared in 100 mM 2-(*N*-morpholino)

ethanesulfonic acid, 1 mM EGTA, and 0.5 mM $MgSO_4$ (pH 6.9). Aliquots of the extracts were adjusted for equal protein content and separated by 12% SDS–PAGE. The separated proteins were then electrophoretically transferred to a nitrocellulose sheet. The sheet was treated sequentially with a Block Ace (Yukijirushi), 500-fold diluted anti-HSV-1 VP16 monoclonal antibody LP1 (McLean *et al.,* 1982), and finally with peroxidase-labeled goat anti-mouse IgG (BioRad, Hercules, CA). The antigen was detected using Renaissance Reagent (DuPont NEN Research Products, Boston, MA) as a substrate.

Virus infection in mice

Transgenic and nontransgenic mice were used when they were 8–10 weeks old. To induce the chimeric gene expression, transgenic mice were injected intraperitoneally with 50 μg of poly(I)–poly(C). Eighteen hours after the induction, the transgenic mice were infected intranasally with 5 μl of PBS containing 2 LD_{50} (viral dose titered on C57BL/6 that was required to produce death in 50% of test animals) of PRV under anesthesia. Deaths of mice were recorded for 14 days. Nontransgenic mice were used as controls.

Anti-PRV antibodies in sera of surviving mice were measured by ELISA (Kida *et al.,* 1982). Disrupted viral antigen was prepared from purified PRV (Ono *et al.,* 1998b).

Virus infection in mouse embryonic fibroblasts

Mouse embryonic fibroblasts were plated at a subconfluent density on 60-mm dishes. The following day, the cells were treated with 1000 U of recombinant mouse interferon-αA/ml for 18 h. After the induction, 0.01 PFU/cell of PRV was adsorbed onto the cells. After 1 h, the cells were washed twice with DMEM and then maintained in 5 ml of DMEM. Supernatant was removed at 24-h intervals. Virus titers of supernatant samples were determined on Vero cell monolayers.

To express PRV IE mRNA selectively, cells that were treated with mouse interferon-αA as described above were treated with cycloheximide (50 μg/ml) for 1 h before infection with PRV at 10 PFU/cell. Infection and maintenance were performed in the presence of the drug. Total cellular RNA was isolated from the cells using an RNA isolation kit (Stratagene Cloning Systems, La Jolla, CA). mRNA isolation was performed using an mRNA separator kit (Clontech, Palo Alto, CA). Purified mRNA samples (10 μg) were electrophoresed on a formaldehyde-containing 1% agarose gel and blot-transferred to a Hybond-N^+ membrane. DIG-labeled riboprobes were derived from pc/IE (Taharaguchi *et al.,* 1994) using the DIG RNA labeling kit. DIG-labeled β-actin probe was purchased from Boehringer Mannheim Biochemica for the detection of β-actin mRNA. Hybridization and detection of PRV IE and β-actin mRNA were performed as described previously by Ono *et al.* (1995).

To detect the fusion protein, mouse embryonic fibroblasts were plated at a subconfluent density onto 100-mm dishes. The following day, the cells were treated with 1000 U of recombinant mouse interferon-αA (Pestka Biomedical Laboratories)/ml for 18 h. After the induction, nuclear extracts were prepared from the mouse embryonic fibroblasts according to the methods of Schreiber *et al.* (1989). Aliquots of the nuclear extracts were adjusted for equal protein content and were applied onto 12% SDS–polyacrylamide gels and electrophoresed. Western blotting was performed as described above.

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